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PRINCIPAL INVESTIGATOR: Gilson S. Baia, Ph.D.

CONTRACTING ORGANIZATION:
Johns Hopkins University

Baltimore, MD 21218-2680

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14. ABSTRACT The aim of our research is to find novel targeted therapy for NF2 patients to provide a new therapeutic option for meningiomas that includes the standard of care radiotherapy. During the first year of this project we have successfully selected four small molecule compounds that preferentially inhibit NF2 mutant meningioma cells <i>in vitro</i> . These are FDA approved drugs that are safe for human use. We validated these drugs by using human cell lines and tested their ability to synergize with radiation. We have optimized the use of a CT-guided conformal radiation that can be tested in a meningioma mouse model. The findings obtained during the first year of this research project will be valuable for second phase, in which we will be testing combinatorial approaches in a meningioma mouse model.					
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INTRODUCTION

Meningiomas are among the most common tumors of the central nervous system and are the second most prevalent tumors in Neurofibromatosis Type 2 patients (Louis, DN *et al* 2007). Despite their high frequency, currently there are no chemotherapeutic options for these tumors and treatment is limited to surgery and various forms of radiation therapy either as adjuvant or primary therapy. Moreover, some tumors are unresectable due to tumor location and have histopathological aggressive features (designated as WHO Grade II and III) with higher recurrence rates. When treatment is recommended, these tumors almost uniformly receive some form of radiation therapy. The aim of this study is to find new therapeutic options to couple with radiation, given its common use with non-surgical meningiomas.

BODY

In this Annual Report we present the research achievements of the first year of the current project. We evaluated a large library of FDA-approved inhibitors to their ability to inhibit meningioma cell growth *in vitro*. Since loss of the NF2 tumor suppressor gene is a major genetic alteration in meningiomas, the library compounds were tested against three pairs of NF2 isogenic cells. Compounds that presented the greatest rates of cell proliferation inhibition were further tested in a secondary screening and also tested for *in vitro* radiation synergy. The best compound-candidates were tested in an orthotopical meningioma mouse model as a single treatment. In addition, in order to accomplish the research plan described on Aim 3 of the project, we optimized the small animal conformal radiation technique applied to the meningioma mouse model. This will allow us to test the combination of both radiation and targeted compounds in the preclinical mouse model, designed for Year 2 of the project.

TASK 1 – Evaluate the in vitro efficacy of combinatorial therapy of small-molecule inhibitors for NF2 mutant meningiomas.

A) Primary drug screen of libraries of FDA-approved compounds. We used cell-based assays to search for small-molecule inhibitors that showed prevalent efficacy against NF2 mutant meningioma cells. Three pairs of NF2 isogenic meningioma cells: AC1, SF6717 and KT21MG1 (Baia, GS *et al*, 2006) were used to screen libraries of FDA approved compounds at a constant final concentration (2 μ M). Briefly, 750 cells/well were plated into 96-well plates and allowed to adhere for 16 hours. Next, 2 μ L of drugs plus 20 μ L of Alamar Blue reagent were added to individual wells. Alamar Blue fluorescence was measured daily over a period of 120h and it was plotted against time. Cells plated with DMSO were used as negative controls. The primary screen was performed using the KT21MG1 cells. We screened a total of 1,726 compounds from 2 libraries (Sigma/LOPAC library and NINDS library: National Institute of Neurological Disorders and Stroke). The top 5% compounds (86 compounds) showing preferential inhibition of NF2 mutant cells were selected for further testing. These compounds were then validated in a second cell proliferation assay that included all three NF2 isogenic cells (3 replicate wells per each compound). Twelve compounds (~15%) showed prevalent inhibitory activity on these NF2 mutant cells. In Table 1 a list of selected compounds is shown. We next determined the half inhibitory concentration (IC₅₀) of the 12 compounds against the NF2 isogenic meningioma cells. A dose-response effect in NF2 mutant cells was observed for most of inhibitors. As seeing in Table 1, a prevalent inhibition of NF2 mutant cells was observed for most of the compounds, compared to the NF2+ cells, as demonstrated by their IC₅₀ values (1.5 to 10-fold in difference).

B) Evaluation of drug-radiation synergy. NF2 isogenic cells (SF6717) were subjected to different doses of radiation (4, 8 and 12Gy) and plated in 96-well plates (1,000 cells/well) for Alamar Blue proliferation assay. Control cells (no radiation) were plated on each plate. Alamar Blue fluorescence data were normalized to the control (0Gy) and plotted against time. As observed in Figure

1, NF2 mutant cells were slightly more sensitive to radiation, compared to NF2 wild type cells. At a 120h time point NF2 mutant cells had a ~25% decrease in cell growth with a radiation dose of 12Gy. For further *in vitro* radiation experiments the dose of 12Gy was chosen to evaluate potential synergist effect with drug combinations. Briefly, for each of the 12 selected drugs, proliferation assays were carried out with cells treated with drugs, radiation (12Gy) or radiation+drugs. Control cells were plated with DMSO. The proliferation data was normalized to the control (DMSO) for each plate and plotted against time (0 to 120h). In Figure 2 the cell proliferation plots of individual drugs testing are shown. Of the tested compounds, an inhibitor targeting the mammalian target of rapamycin, mTOR (Everolimus), 2 inhibitors targeting the vascular endothelial growth factor receptor, VEGFR (Tandutinib, Sunitinib) and Gleevec presented the greatest synergistic effect of drugs in combination with radiation. Moreover, the cell proliferation inhibition was significant at 120h for all 4 compounds ($P \leq 0.05$). The other 8 inhibitors either presented either additive effect or no effect in combination with radiation.

C) Secondary drug screening. Next, we tested whether the combination of drug and radiation affected the anchorage-independent growth, a phenotype characteristic of tumor cells. Briefly, heavily irradiated (50Gy) IOMM-Lee cells were plated as feeder cells in 6-well plates (30,000 cells/well). Twenty four hours later, 600 experimental NF2 mutant cells were plated in triplicate wells. Plates were incubated for 7 to 10 days until visible colonies were observed. Cells were washed in PBS, fixed in 10% formalin and stained with crystal blue. Colonies of >50 cells were counted under a dissecting microscope. As shown on Figure 3, all combinations of drug and radiation treatment formed significantly fewer colonies compared to either controls (radiation or drug alone).

Together, these data demonstrate that in combination with radiation the inhibitors: Everolimus, Gleevec, Sunitinib and Tandutinib are promising for potential for therapeutic treatments of NF2 mutant meningioma cells.

TASK 2 – Determine the potential survival benefit of combinatorial therapy of small-molecule inhibitors in an orthotopic meningioma mouse model.

A) Preclinical testing of individual selected drugs. We employed an orthotopic meningioma mouse model to determine the potential efficacy of drugs in combination with radiation. The goal was to evaluate the potential survival benefit of combinatorial therapy *in vivo*. Briefly, mice intracranial implantations were performed in accordance with an animal protocol approved by the Johns Hopkins Institutional Animal Care and Use Committee. NF2 mutant cells were implanted orthotopically into athymic mice, as previously described (Baia, GS *et al*, 2008). Briefly, 6 week-old female athymic mice were anesthetized (Ketamine 80mg/Kg; Xylazine 10mg/Kg) and fixed in a stereotaxic frame. Cells (10^5 in 1 μ l) were implanted into the floor of temporal fossa by using the following stereotaxic coordinates relative to the bregma: 2 mm to the right, 2 mm posterior and 6 mm of depth. Figure 4A illustrates the tumor implantation site. Meningioma cells were tagged with a firefly Luciferase construct, under the control of the spleen focus forming virus promoter, via lentiviral transfection, as previously described (30, 31). Bioluminescence imaging (BLI) was used to monitor tumor growth progression. Figure 4B shows a representative BLI image of a mouse at day 5 post-cell transplantation. After cell implantation, mice were appropriately monitored three times every week and euthanized if they exhibit neurological symptoms or had more than 15% of weight loss. NF2 mutant cells were tumorigenic in Athymic nude mice (Figure 4C).

The drugs Everolimus and Gleevec, selected on Taks#1 were chosen to begin the preclinical testing because they present very high *in vivo* tolerance at the equivalent maximum tolerated dose (MTD) in humans. NF2 mutant cells were implanted as described above and at day 3 animals were treated daily, for 5 days a week (Monday-Friday) with either Everolimus (2mg/Kg) or Gleevec (100mg/Kg). Tumor bearing mice were used as controls (no treatment). Animal survival was plotted overtime (Figure 6). As observed, neither of the single agents was capable to prolong survival of NF2 mutant xenografts. We anticipate

testing next, both combinations of drugs and radiation+drugs to evaluate potential beneficial survival of meningioma xenografts.

B) Optimization of small animal conformal radiation testing. To evaluate the synergistic effect of drugs and radiation (Aim 3/Year 2) we plan to employ the Small Animal Radiation Research Platform (SARRP) to precisely deliver localized radiation to xenografts. To optimize the experiment we run a pilot study. Briefly, NF2 mutant meningioma cells were implanted orthotopically into mice (10^5 cells/mouse). Three days after cells implantation mice were anesthetized and treated once with localized radiation (10, 12 or 20 Gy), using an in-house developed precision small animal radiation device (Wong, J *et al*, 2008). This technology is capable of delivery of high intensity and localized doses of radiation to the target tumor volume, while minimally affecting the surrounding normal tissue. Since radiosurgery is part of standard of care of meningioma patients, this CT-guided conformal radiation is pivotal in mimicking clinical radiotherapy. Treated mice showed longer survival times compared to control mice. Figure 6 shows a Kaplan-meier survival plot of mice treated with 12Gy (n=6) compared to control group (n=6). Radiation treated mice had a slightly longer survival compared to control mice. We anticipate using this radiation approach to test the efficacy of drug treatments in meningioma xenografts, as planned for the Year 2 of this project.

KEY RESEARCH ACCOMPLISHMENTS

- 1- We have screened large libraries of FDA approved compounds searching for small-molecule inhibitors that preferentially inhibit NF2 mutant meningiomas.
- 2- We validated a set of selected 12 compounds by screening these drugs in 2 additional pairs of cells.
- 3- We tested the set of 12 compounds for synergy effect with radiation. Four compounds showing the greatest synergy with radiation were selected for further testing.

- 4- The colony forming efficiency assay was performed as a secondary screening to test the inhibitory efficiency of selected drugs in combination with radiation.
- 5- We tested the *in vivo* efficacy of Everolimus and Gleevec in a meningioma mouse model.

REPORTABLE OUTCOMES

- Research data presented at the 103rd American Association for Cancer Research Annual Meeting, Washington D.C., USA, 2012 (Poster format, Figure 7).
- Optimized the use of CT-guided conformal radiation applied to the meningioma mouse model.

CONCLUSIONS

Using a cell-based assay we have successfully selected four small molecule compounds that preferentially inhibit NF2 mutant meningioma cells *in vitro*. These are FDA approved drugs that are safe for human use. We validated these drugs by using human cell lines and tested their ability to synergize with radiation. We have entered the second phase of the research project to find a window of opportunity for treatment of meningiomas. Our goal is to accelerate clinical trials designed for NF2 associated meningiomas and to find better therapeutic options that can improve the quality of life for NF2 patients.

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APPENDICES

Table 1 – IC₅₀ values (μM) determined for NF2 isogenic cells. Drugs were selected for preferential inhibition of NF2 mutant cells from primary drug screening of 1,726 compounds.

	KT21MG1		SF6717		AC1	
	NF2-	NF2+	NF2-	NF2+	NF2-	NF2+
VEGFR/PDGFR						
Sorafenib (Nexavar)	3.7μM	5.7μM	0.9μM	3.2μM	1μM	3μM
Sunitinib (Sutent)	2.3μM	2.8μM	2.7μM	4.7μM	3.9μM	3.1μM
Tandutinib (MLN518)	15.3μM	20.4μM	47μM	45.8μM	41μM	41μM
EGF Receptors						
Lapatinib (Tyrkeb)	3.8μM	5.2μM	3μM	4μM	2.5μM	4μM
Gefitinib (Iressa)	18μM	22μM	22μM	24μM	17μM	22μM
BCR-ABL inhibitors						
Imatinib (Gleevec)	16μM	23.3μM	13.7μM	39.7μM	13.8μM	18.3μM
mTOR/PI3K						
BEZ235	0.6nM	2.8nM	0.6nM	1.2nM	0.9nM	1.8nM
Rapamycin	1μM	2.2μM	0.6μM	1.3μM	1μM	3.5μM
Everolimus (Afinitor)	23μM	26.5μM	17.4μM	20.6μM	18μM	20μM
Anti-parasitic						
Emetine	0.04μM	0.06μM	0.02μM	0.04μM	0.02μM	1μM
Topo Inhibitors						
Idarubicin	8.5nM	85.5nM	0.3μM	0.1μM	x	x
Mitoxantrone	7μM	8.2μM	5μM	10μM	x	x

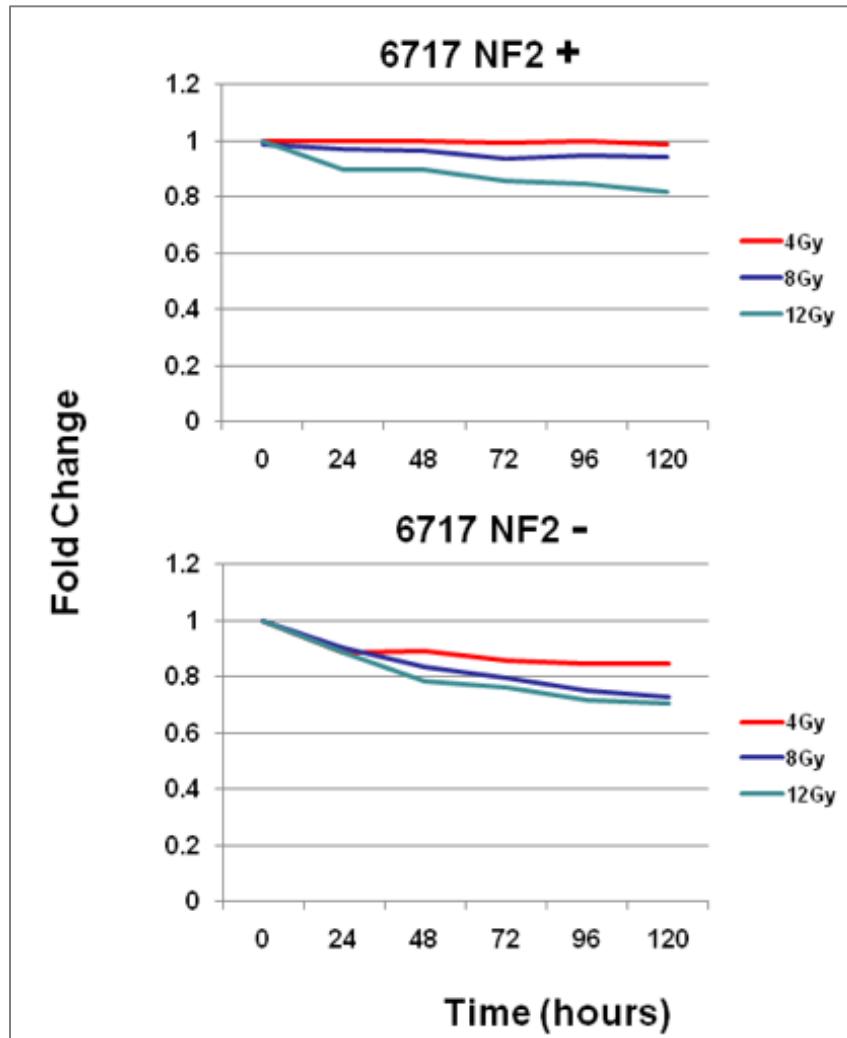


Figure 1 – *In vitro* Alamar Blue proliferation assay of wild type and mutant NF2 cells treated with different doses of radiation. Cell proliferation was normalized to the control cells (0Gy) and is plotted over time.

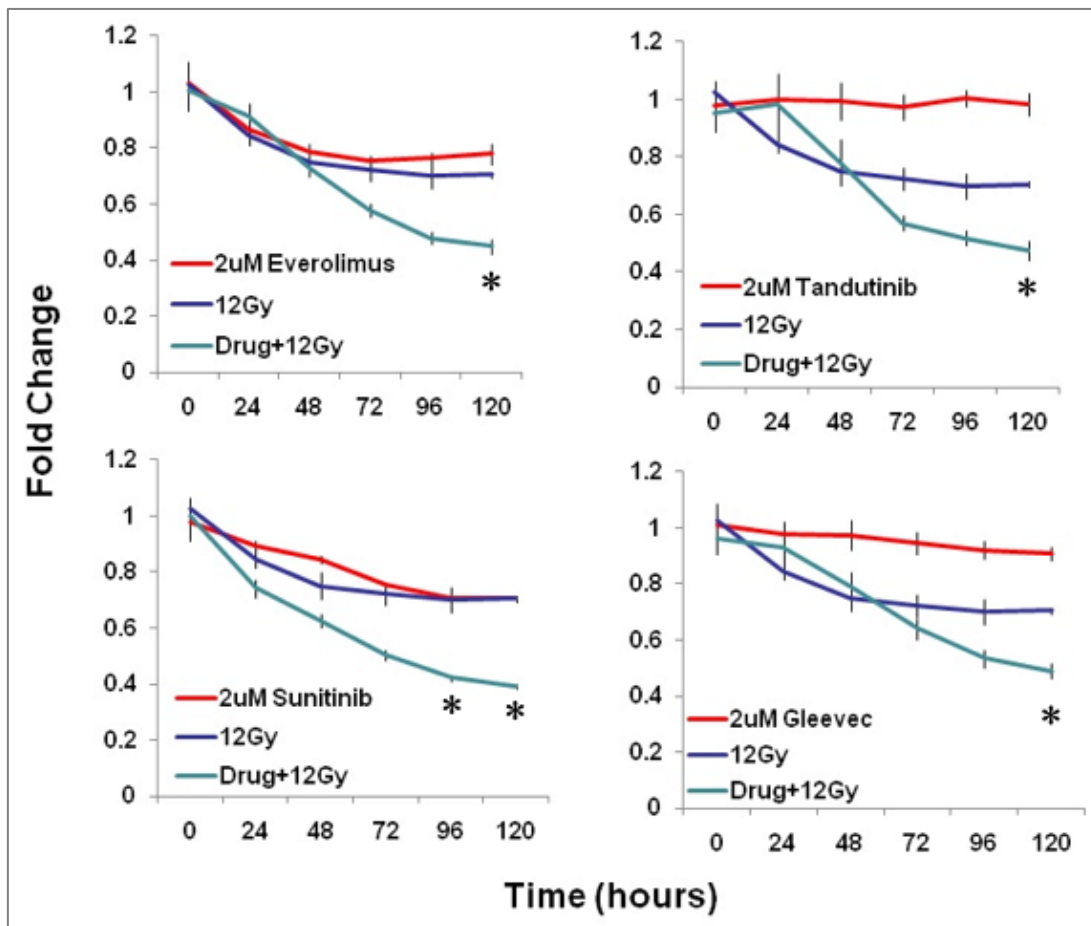


Figure 2 – Synergistic inhibition of drugs with radiation (12Gy) in KT21MG1 mutant cells. Proliferation assays were carried out over 120h with cells. Proliferation assays were carried out over 120h with cells treated with drugs (2 μ M), radiation (12Gy) or drugs+radiation. Synergistic effect of drugs in combination with radiation was observed for Everolimus, Tandutinib, Sunitinib and Gleevec (* $P \leq 0.05$).

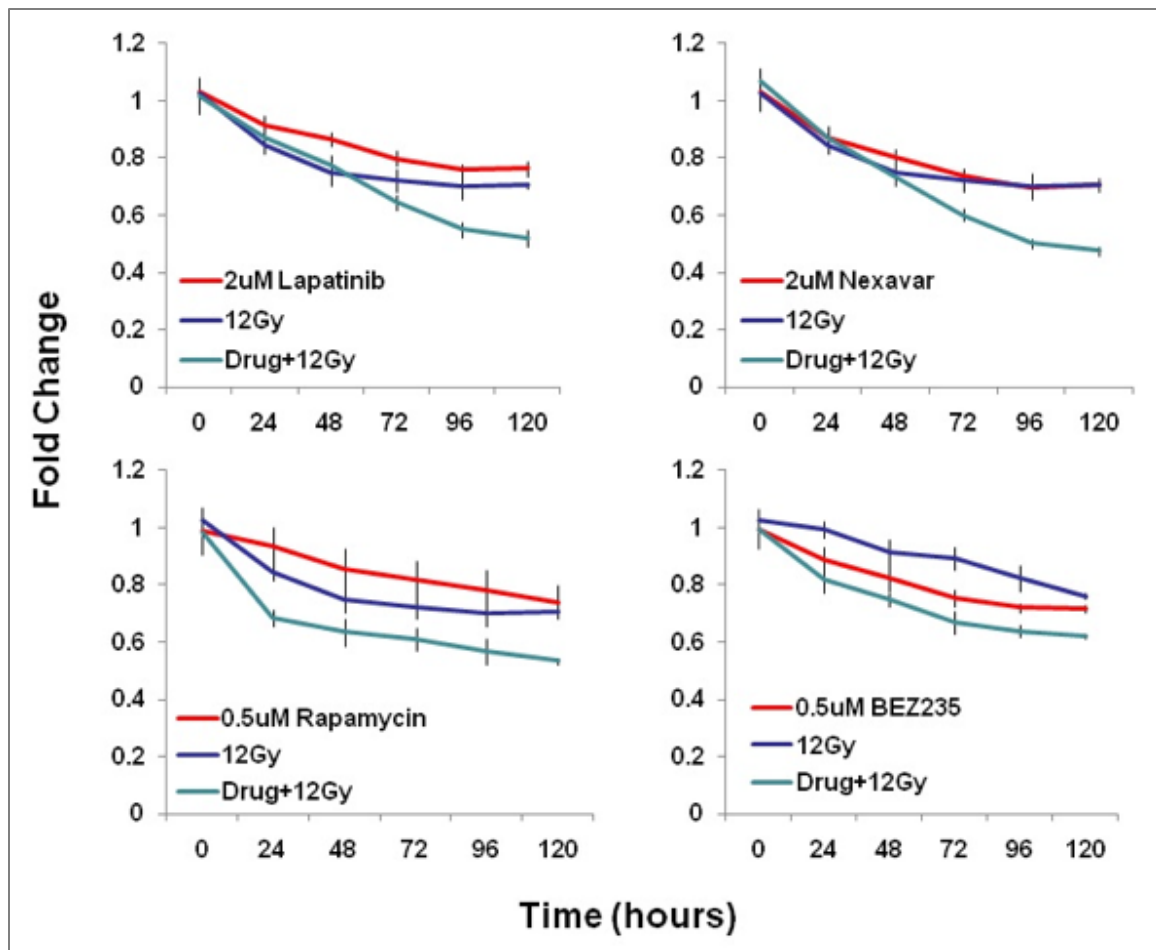


Figure 2 – Synergistic inhibition of drugs with radiation (12Gy) in KT21MG1 mutant cells. (Continued).

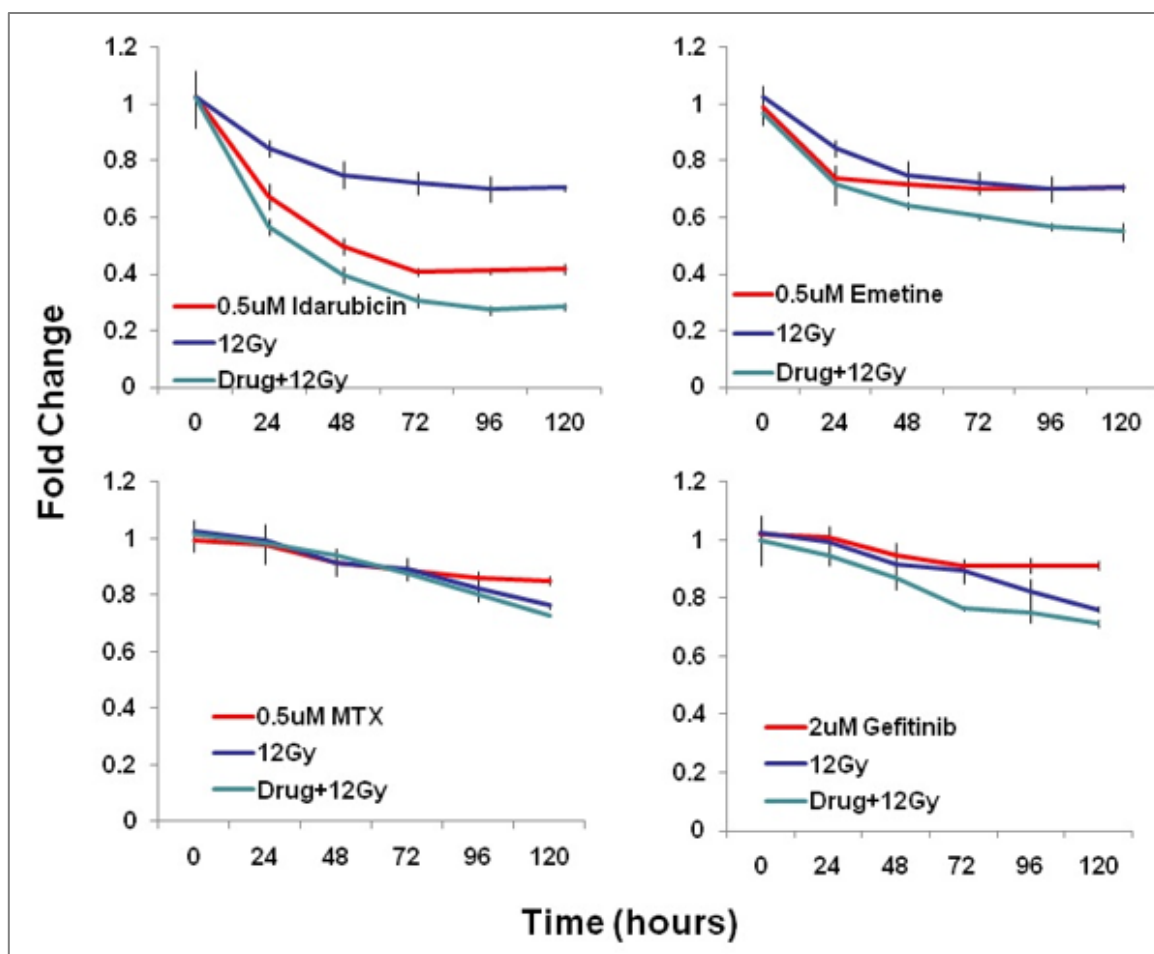


Figure 2 – Synergistic inhibition of drugs with radiation (12Gy) in KT21MG1 mutant cells. (Continued).

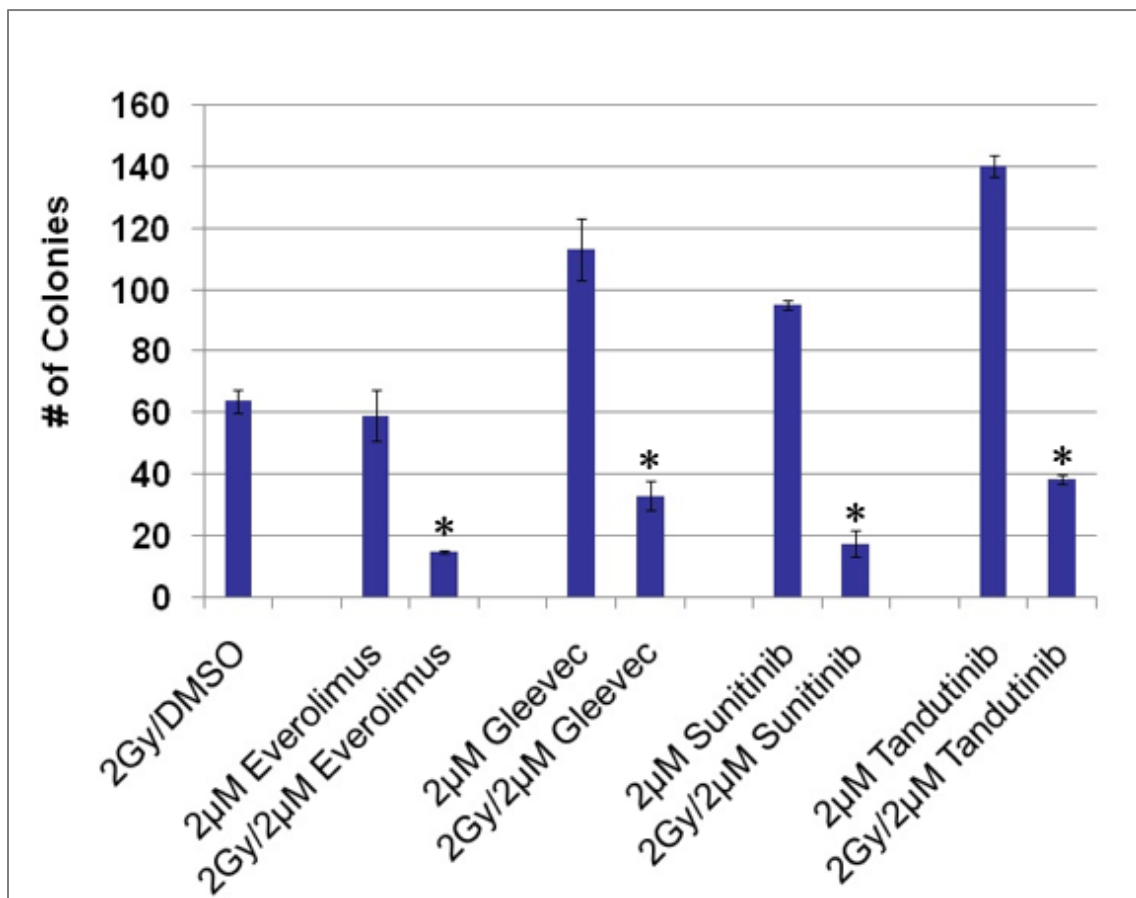


Figure 3 – Secondary drug/radiation screening – Colony-forming efficiency assay was performed with NF2 mutant cells treated with drugs and radiation. Cells treated with both drug and radiation together formed significantly fewer colonies compared to the controls with either radiation or drugs alone ($P \leq 0.05$).

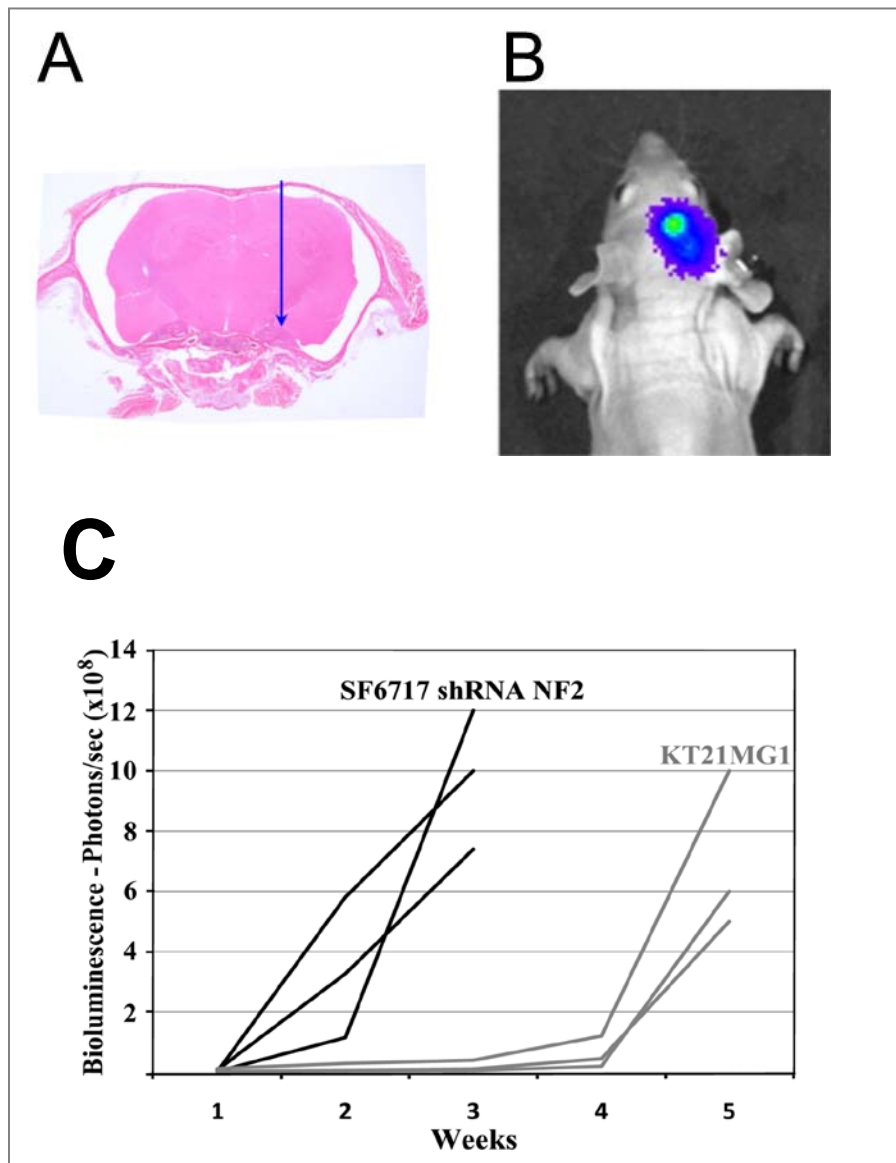


Figure 4 – NF2 mutant cells are tumorigenic *in vivo* in Athymic nude mice. A) Tumor implantation site (arrow) shown on a coronal section of a mouse brain. Meningioma cells were implanted into the floor of temporal fossa by using the following stereotaxic coordinates relative to the bregma: 2 mm to the right, 2 mm posterior and 6 mm of depth. B) Bioluminescence (BLI) of Firefly Luciferase tagged cells was used to monitor tumor growth. A BLI image of a representative animal is shown at day 5 after cell implantation. C) Meningioma cells were orthotopically implanted into nude mice. NF2 mutant cells were tumorigenic in nude mice as shows by the BLI measurements over time (weeks).

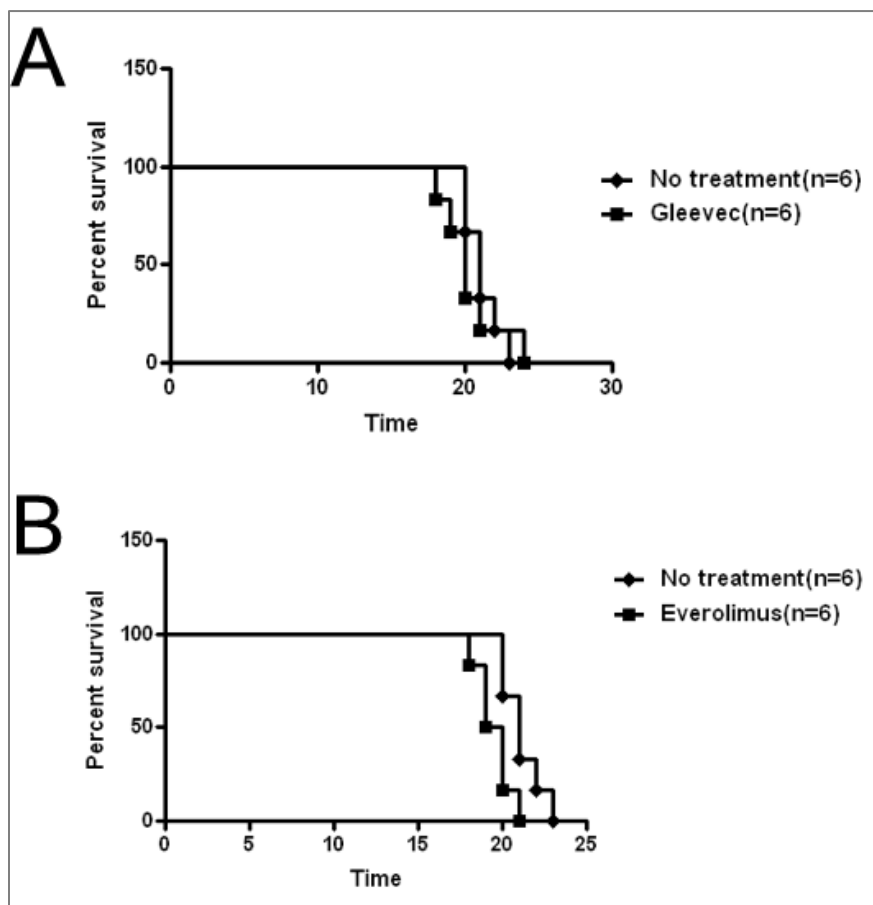


Figure 5 – Kaplan-meier survival plots of NF2 mutant meningioma xenografts. Mice were treated with either Everolimus (A) or Gleevec (B) compared to control animals (no treatment). (Time in days).

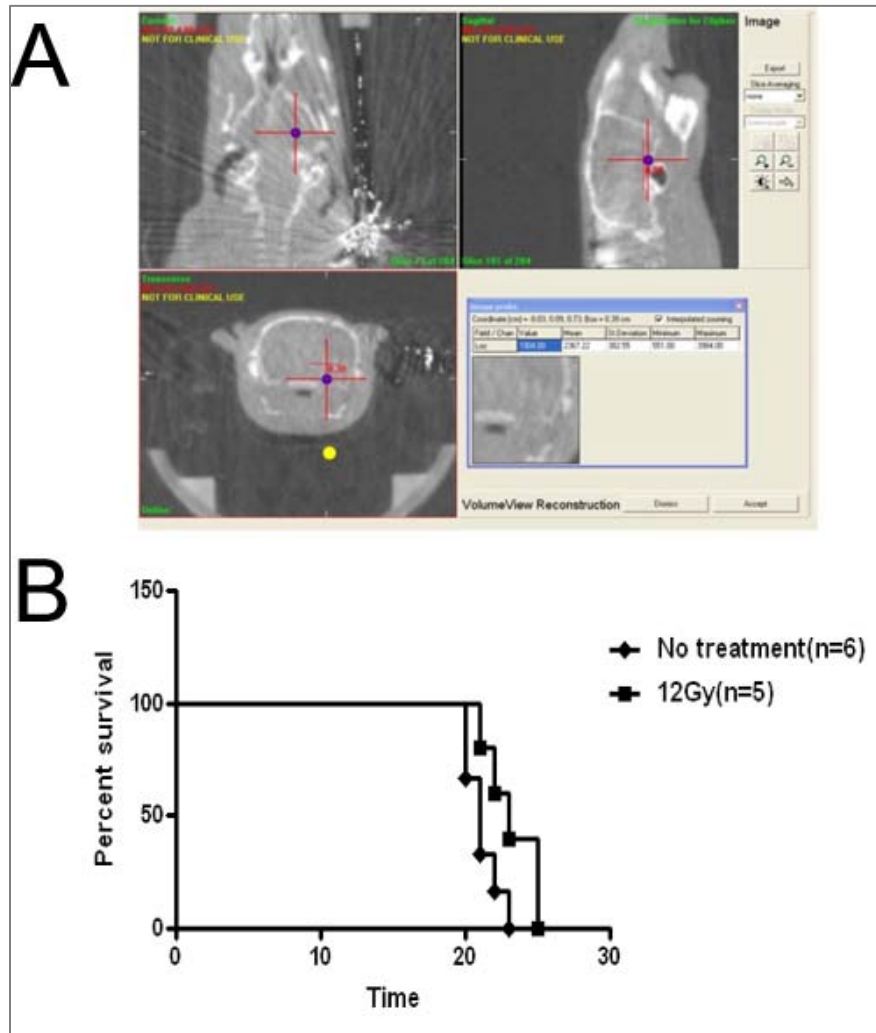


Figure 6 – Stereotaxic radiosurgery device. NF2 mutant meningioma cells were implanted orthotopically into mice (10^5 cells/mouse). Three days after cell implantation mice were anesthetized and treated once with conformal radiation (12Gy), using an in-house developed precision small animal radiation device. This technology is capable of delivering high intensity and localized doses of radiation to the target tumor volume, while minimally affecting the surrounding normal tissue. Since radiosurgery is part of the standard of care of meningioma patients, this CT-guided conformal radiation is pivotal in mimicking clinical radiotherapy (A). Mice survival plots (B) of animals treated with radiation (12Gy) compared to the non-treated controls. (Time in days).

Figure 7 – Poster presented at the 103rd American Association for Cancer Research Annual Meeting, Washington D.C., USA, 2012.

Targeting NF2 deficient meningiomas with combinations of small molecule inhibitors and radiation. Gilson S. Baia, Graeme Woodworth, Eric Ford, Gregory Riggins. Johns Hopkins, Baltimore, MD

Poster Session - PO.ET01.01. Combination Therapy 1 -Mon, Apr 2, 1:00 - 5:00 PM

(Figure on next Page)



Targeting NF2 Deficient Meningiomas with Combinations of Small Molecule Inhibitors and Radiation

Gilson S. Baia, PhD¹; Graeme Woodworth MD¹; Eric Ford PhD²; Gregory J. Riggins MD PhD¹

¹Department of Neurosurgery and ²Radiation Oncology Department

Johns Hopkins University School of Medicine, Baltimore, MD USA



BACKGROUND

Meningiomas are common tumors of the central nervous system and are the second most prevalent tumors in Neurofibromatosis type 2 patients. Despite their high frequency, currently there are no chemotherapeutic options for these tumors and treatment is limited to surgery and various forms of radiation therapy either as adjuvant or primary therapy. Some tumors are unresectable due to location and have histopathological aggressive features (designated as WHO grade II and III) with higher recurrence rates. When treatment is recommended, these tumors almost uniformly receive some form of radiation therapy. The aim of this study was to find new therapeutic options to couple with radiation, given its common use with non-surgical meningiomas. We are interested in targeting oncogenic pathways in the context of loss of the NF2 gene, which represents the most common genetic alteration in meningiomas, present is 50-70% of sporadic tumors and all of NF2 cases. We used cell-based assays searching for small molecule inhibitors that showed prevalent efficacy against NF2-deficient cells. Cell proliferation and clonogenic assays were employed to investigate the potential synergy effect of drugs and radiation combinations. Three pairs of NF2 isogenic meningioma cells (AC1, SF6717 and KT21MG1) were used to screen libraries of FDA approved compounds. The primary screen was performed using KT21MG1 cells. The top 5% compounds (86 compounds) showing preferential inhibition of NF2-deficient cells were selected for further validation. The secondary screen was used to validate useful targets with AC1 and SF6717 cells. Twelve compounds (~15%) showed prevalent inhibitory activity on these cells. Small molecules targeting mammalian target of rapamycin (mTOR) and vascular endothelial growth factor receptor (VEGFR) showed preferential synergistic inhibition of NF2 cells with radiation, compared to either radiation or drug treatment alone. Currently, preclinical testing is in progress to investigate the efficacy of these inhibitors in combination with radiation in a meningioma mouse model.

STUDY DESIGN

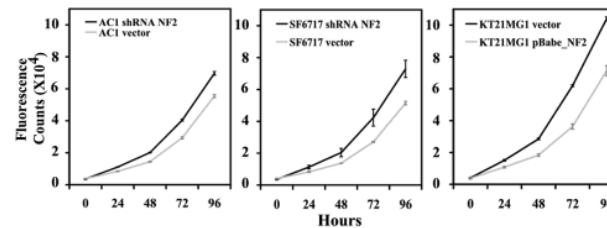
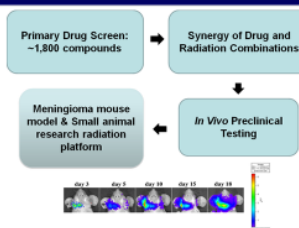


Figure 1 - Loss of Tumor Suppressor NF2 Promotes In Vitro Cell Proliferation. We determined the cell proliferation rates of NF2 isogenic cell lines. As expected, loss of the tumor suppressor NF2 conferred gain of cell proliferation, as measured by the Alamar Blue reagent assay (Invitrogen). These NF2 isogenic cells were further used to screen libraries of FDA approved drugs.

Table 1 – IC50 Values Determined for NF2 Isogenic Cells. Drugs were selected for preferential inhibition of NF2 mutant cells.

	NF2-	NF2+
Sorafenib (Nexavar)	3.78uM	6uM
Sunitinib (Sutent)	2.3uM	3uM
Vandetanib (Zactima)	5.2uM	4uM
Pazopanib (Votrient)	3uM	3uM
Tandutinib (MLN518)	15.3uM	20.4uM
Gefitinib (Iressa)	13.5uM	10uM
Erlotinib (Tarceva)	120uM	120uM
Nilotinib (Tasigna)	2uM	1.7uM
Dasatinib (BMS-354825)	23uM	17uM
NVP-BE235	0.5nM	3nM

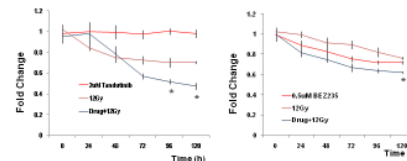


Figure 2 – Synergistic Inhibition of Drugs with Radiation in KT21MG1 NF2 mutant Cells. Proliferation assays were carried out over 120h with cells treated with Drugs, Radiation or Drugs+Radiation. Synergistic effect of drugs in combination with radiation was observed for BE235 and Tandutinib ($P=0.05$).

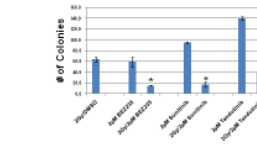


Figure 3 – Secondary Drug/Radiation Screening. Colony-forming efficiency assay was performed with KT21MG1 NF2 mutant cells treated with drugs and radiation. Cells treated with both drug and radiation together formed significantly fewer colonies compared to the controls with either radiation or drugs alone ($P=0.05$).

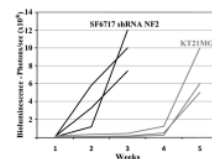


Figure 4 - NF2 Deficient Cells Are Tumorigenic In Vivo. Meningioma cells were orthotopically implanted into nude mice and bioluminescence was used to monitor tumor progression. NF2 mutant cells were tumorigenic in nude mice.

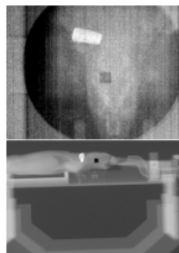


Figure 5 - Stereotaxic Radiosurgery Device. NF2 mutant meningioma cells were implanted orthotopically into mice (250,000 cells/mouse). Three days after cells implantation mice were anesthetized and treated once with localized radiation (20Gy), using an in-house developed precision small animal radiation device. This technology is capable of delivery of high intensity and localized doses of radiation to the target tumor volume, while minimally affecting the surrounding normal tissue. Since radiosurgery is part of standard of care of meningioma patients, this CT-guided conformal radiation is pivotal in mimicking clinical radiotherapy.

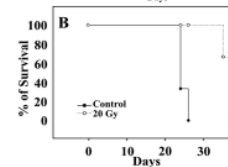
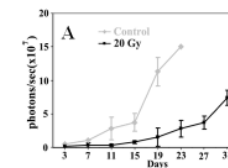


Figure 6 - Radiosurgery of meningioma implanted mice. A) Bioluminescence plot (BLI) of 3 mice/group averaged for each time point associated with monitoring of intracranial tumor growth and B) survival times of radiation treated mice and control group ($p<0.0735$). Treated mice showed much lower bioluminescence reads, compared to control groups (Fig. A), demonstrating that the radiation treatment is capable of slowing down tumor growth. In addition, treated mice had longer survival times compared to control mice (Fig. B).

CONCLUSIONS

- Small molecule inhibitors preferentially affecting NF2 mutant cells were selected.
- Compounds synergizing with radiation were selected for further pre-clinical testing in nude mice.
- CT-guided conformal radiation was optimized for NF2 mutant cells.

FUTURE DIRECTIONS

- Preclinical testing is in progress to investigate the efficacy of selected small molecule inhibitors in combination with conformal radiation in a NF2 mutant meningioma mouse model.

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